METHODS

Subjects

Exclusion criteria were general maladies, severe skin inflammation, pregnancy and/or lactancy, autoimmune diseases, contraindication for adrenaline, long-term use of systemic corticosteroids, intake of immunosuppressive or psychoactive drugs, a positive SPT response to hypoallergenic Bet v 1 fragments, simultaneous participation in another clinical study, and risk of noncompliance with study procedures and restrictions. Use of antihistamines within the previous 3 days and use of systemic short-term and topical corticosteroids in the tested area within the previous 14 days were not allowed. Participants signed informed consent forms before inclusion in the study.

Study materials, SPTs, and APTs

SPTs were conducted with commercial birch pollen extract (Stallergenes, Antony, France), purified rBet v 1, and 2 hypoallergenic rBet v 1 fragments (F1 and F2 each or as the equimolar mix F1+F2), which together comprise the complete Bet v 1 sequence.[E1] Concentrations were 20 and 40 μg/mL (rBet v 1, each of the fragments), as well as an equimolar mix of F1+F2 containing 10 and 20 μg/mL of each fragment. Aliquots of 20 μL were applied in duplicates on the left and right side of the forearm of the subjects at a distance of more than 2 cm between individual application points. As controls, 1 mg/mL histamine hydrochloride (positive) and sodium chloride solution (negative, Stallergenes) were used. After 20 minutes, wheal-and-flare reactions were photodocumented, pen marked, and transferred with scotch tape to a paper. The mean wheal area of duplicate tests was calculated by using digital planimetry. Only wheals of more than 4 mm in diameter were regarded as positive reactions.

For APTs, birch pollen extract (Stallergenes), as well as rBet v 1 (160 μg), rBet v 1 fragment 1 (160 μg), rBet v 1 fragment 2 (160 μg), and an equimolar rBet v 1 fragment mix (80 μg of each rBet v 1 fragment), were applied for 48 hours in patch test chambers (12 mm in diameter; Finn Chambers on Scanpor, Large, Epitest Ltd Oy) onto nonlesional skin on the backs of the subjects. The skin of the subjects was stripped shortly before with a tape. A patch containing pure Vaseline petroleum jelly (Unilever) was used as a negative control. After 48 hours, patches were removed, and reactions were analyzed and photodocumented. Grading of positive APT reactions were done according the European Task Force on Atopic Dermatitis by a blinded investigator: -, negative result; ?, only erythema, questionable; +, erythema, infiltration; ++, erythema, few papules (≤3); +++, erythema, papules from 4 to less than many; ++++, erythema, many or spreading papules; or +++++, erythema, vesicles.[E2]

Total and specific IgE levels

Nitrocellulose strips (Whatman Protran nitrocellulose membrane; Sigma-Aldrich, St Louis, Mo) containing 2-μL aliquots (ie, 1 μg of rBet v1, rBet v 1 fragment 1, rBet v 1 fragment 2, an equimolar mix of the rBet v 1 fragments 1 and 2 [ie, 0.5 μg each], and BSA) were incubated overnight with sera from the 30 study participants (1:10 in gold buffer) or with buffer without addition of serum. IgE reactivity was determined, as previously described.[E3] Signals obtained from the dot blots were quantified by means of densitometry with National Institutes of Health ImageJ software analysis, as described previously.[E4]

Lymphocyte proliferation assays and detection of secreted cytokines

PBMCs were isolated from heparinized blood samples by means of Ficoll density gradient separation (Amersham, GE Healthcare, Buckinghamshire, United Kingdom). PBMCs were stimulated with 5 μg/well of rBet v 1 or an equimolar mix of hypoallergenic rBet v 1 fragments (F1+F2) in triplicates at a density of 2×10^6 cells/well in 96-well round-bottom plates (Thermo Fischer Scientific, Roskilde, Denmark). Cells were cultured in 200 μL of Ultra Culture Medium (Lonza, Verviers, Belgium) supplemented as previously described.[E5] Wells containing 4 units of human IL-2 each (Roche Diagnostics GmbH, Mannheim, Germany) or medium alone were used as positive and negative controls, respectively. Cells were incubated for 6 days in a humidified atmosphere containing 5% CO_2 at 37°C. Proliferation was measured based on tritiated thymidine uptake (0.5 μCi/well; PerkinElmer, Boston, Mass) after 16 hours of culture. Radioactivity was measured in counts per minute. The results were displayed as the stimulation index, which was calculated as the quotient of counts per minute in stimulated and unstimulated cultures. A response was considered positive when the stimulation index was greater than 1.

Supernatants from equally prepared PBMC cultures were harvested at day 6, and IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, INF-γ, TNF-α, C-GSF, GM-CSF, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1 levels were measured with the Bio-Plex Pro human cytokine 17-plex immunoassay (Bio-Rad Laboratories, Hercules, Calif). In addition, IL-22 levels were measured in each sample by using the FlowCytomix Human IL-22 Simplex Kit (eBioscience, San Diego, Calif). Cytokine values were log transformed before statistical analysis because their distributions were asymmetric.

Flow cytometric analysis of CLA^+ and $CCR4^+$ T cells

PBMCs were isolated as described above and stained with carboxyfluorescein diacetate succinimidyl (Invitrogen, Oslo, Norway; 1 mL of 5 μmol/L carboxyfluorescein diacetate succinimidyl ester solution per 10×10^6 cells) for 10 minutes at 37°C. The labeling reaction was stopped by adding FBS (GIBCO, Invitrogen, Carlsbad, Calif) for 5 minutes. Labeled cells were washed with medium and cultured in triplicates (2×10^6 cells/well) with rBet v 1 (5 μg/well), equimolar mix of hypoallergenic rBet v 1 fragments (F1+F2), medium alone (negative control; Lonza, Verviers, Belgium), or 3 μL/well of Dynabeads containing anti-CD3 and anti-CD28 (positive control, Invitrogen) for 7 days at 37°C. At day 7, cells were centrifuged and incubated in 50 μL of solution containing 7-amino-actinomycin D (3 μL/well; BioLegend, San Diego, Calif) plus 10 μL/well of biotin mouse anti-human CD194 (Anti-CCR4) or biotin rat anti-CLA (BD Biosciences, San Jose, Calif) diluted in fluorescence-activated cell sorting (FACS) buffer (PBS, 0.01% wt/vol NaN_3, and 1% wt/vol BSA) for 20 minutes on ice. For control purposes, a biotin rat IgM isotype (CLA isotype) and a mouse IgG_1 isotype (CCR4 isotype, BD Bioscience) were used. Cells were then centrifuged and stained with 1 μL/well of streptavidin-phycoerythrin-cyanine 7 (PC7-Streptavidin, BD Biosciences) diluted in FACS buffer for 20 minutes on ice. After centrifugation, cells were resuspended in FACS buffer and measured with a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, Calif). Data analysis was done with FlowJo Version 7.2.5 (TreeStar, Ashland, Ore). The gating strategy was based on forward and side scatter. 7-Amino-actinomycin D (BioLegend) was used for dead-cell exclusion. Mean percentages of CLA^+CD3^+ and $CCR4^+CD3^+$ T cells were calculated. The results were presented as stimulation indices calculated as the quotient of mean percentages of $CD3^+$ T cells expressing CLA or CCR4 in stimulated and medium-only cultures.

Basophil activation by means of flow cytometry: CD203c and CD63 assay

Peripheral blood was obtained from a donor in heparinized tubes after informed consent was provided. Blood aliquots (100 μL) were incubated (triplicates) for 15 minutes at 37°C with 10-μL supernatants from PBMC cultures stimulated with rBet v1, rBet v 1 F1+F2, and medium and then washed in PBS containing 20 mmol/L EDTA (Gibco). Anti-IgE mAb E-124.2.8 (1 μg/mL) or PBS were used as controls. Thereafter, cells were incubated with 5 μL of phycoerythrin-conjugated CD203c mAb 97A6 and 5 μL of fluorescein isothiocyanate–labeled CD63 mAb CLB-gran12 for 15 minutes at room temperature. After erythrocyte lysis with FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, Calif), cells were washed, resuspended in PBS, and analyzed by means of 2-color flow cytometry on a FACScan (Becton Dickinson Biosciences) with FlowJo software (Tree Star). Anti-IgE–induced upregulation of CD203c/CD63 was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated ($MFI_{control}$) cells and expressed as the stimulation index (MFI_{stim}:$MFI_{control}$).[E6]

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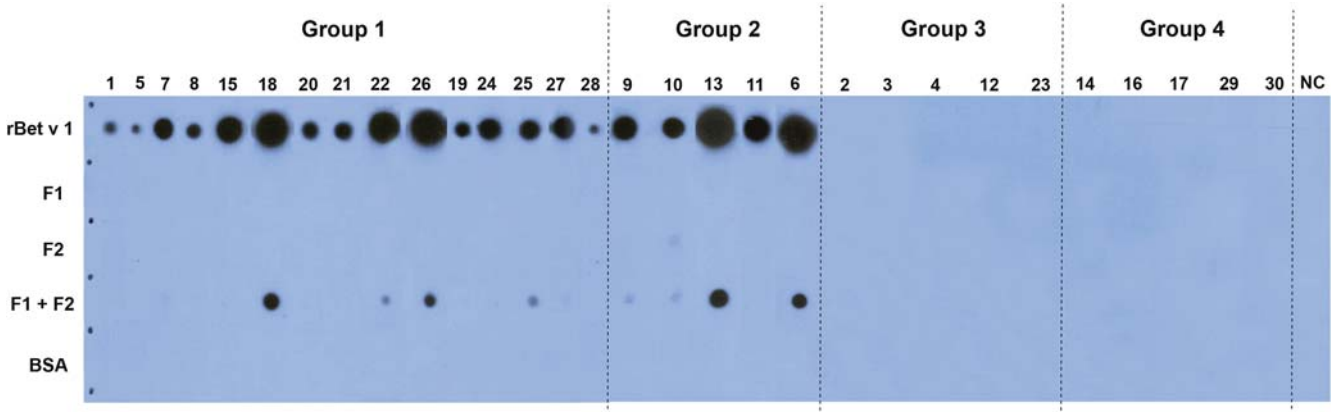

FIG E1. IgE reactivity to rBet v 1 and rBet v 1 fragments. Dot-blotted purified recombinant antigens (rBet v 1, rBet v 1 fragments F1 and F2, an rBet v 1 fragment mix [F1+F2], and BSA) were incubated with sera from the study subjects from groups 1 to 4 *(1-30*) or with buffer alone as a negative control *(NC)*. Bound IgE antibodies were detected with iodine 125–labeled anti-human IgE antibodies and visualized by means of autoradiography.

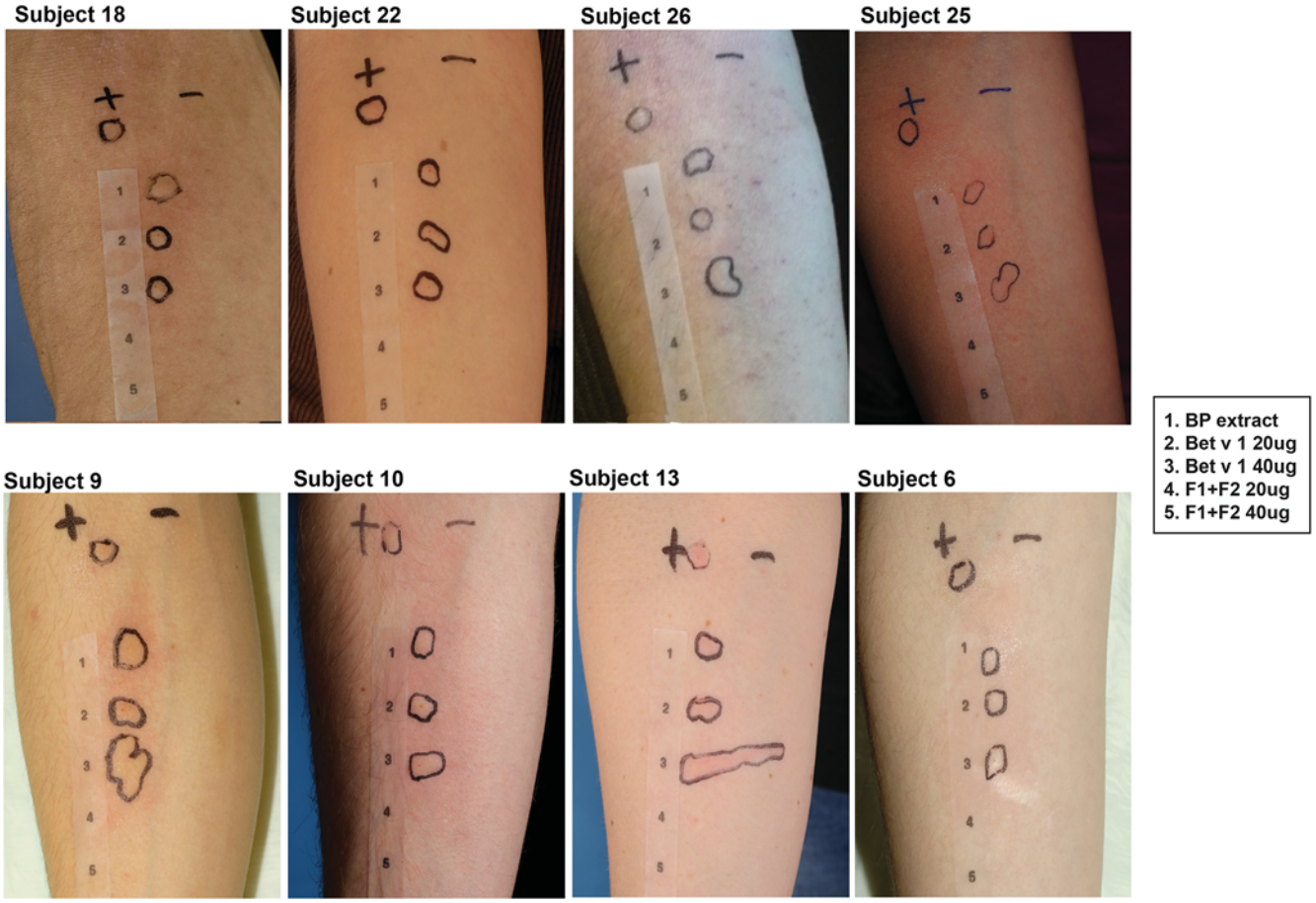

FIG E2. Immediate-type skin reactions to rBet v 1 and rBet v 1 fragments in subjects with residual IgE reactivity to the F1+F2 mix in RAST-based dot blotting assay. SPTs were performed with birch pollen extract *(1)*, rBet v 1 (*2* and *3*), and the mix of rBet v 1 fragments F1+F2 (*4* and *5*). SPTs were performed with antigen concentrations of 20 μg/mL (*2* and *4*) or 40 μg/mL (*3* and *5*).

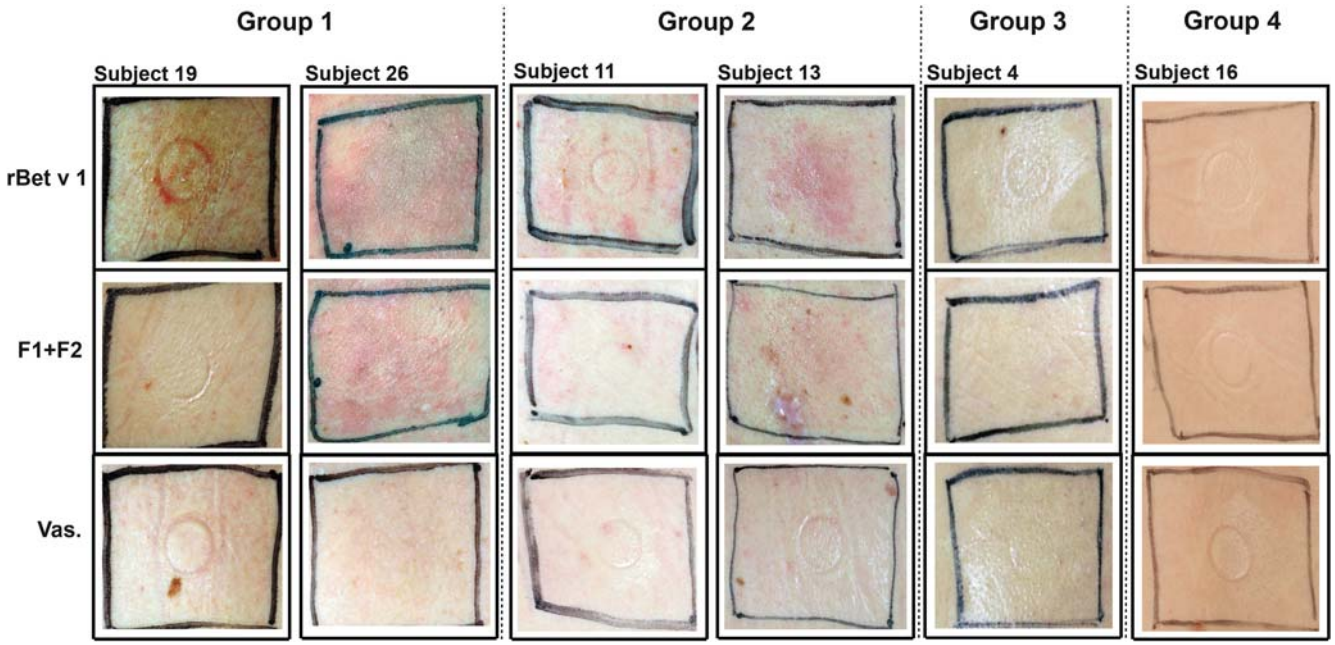

FIG E3. Delayed-type skin reactions to rBet v 1 and rBet v 1 fragments in selected subjects. Shown are APT reactions to rBet v 1 and the rBet v 1 fragment mix (F1+F2) in patients from study groups 1 to 4 (group 1, subjects 26 and 19; group 2, subjects 13 and 11; group 3, subject 4; and group 4, subject 16). APTs were performed with 160 μg of rBet v 1 and a mix containing 80 μg of each rBet v 1 fragment (F1+F2).

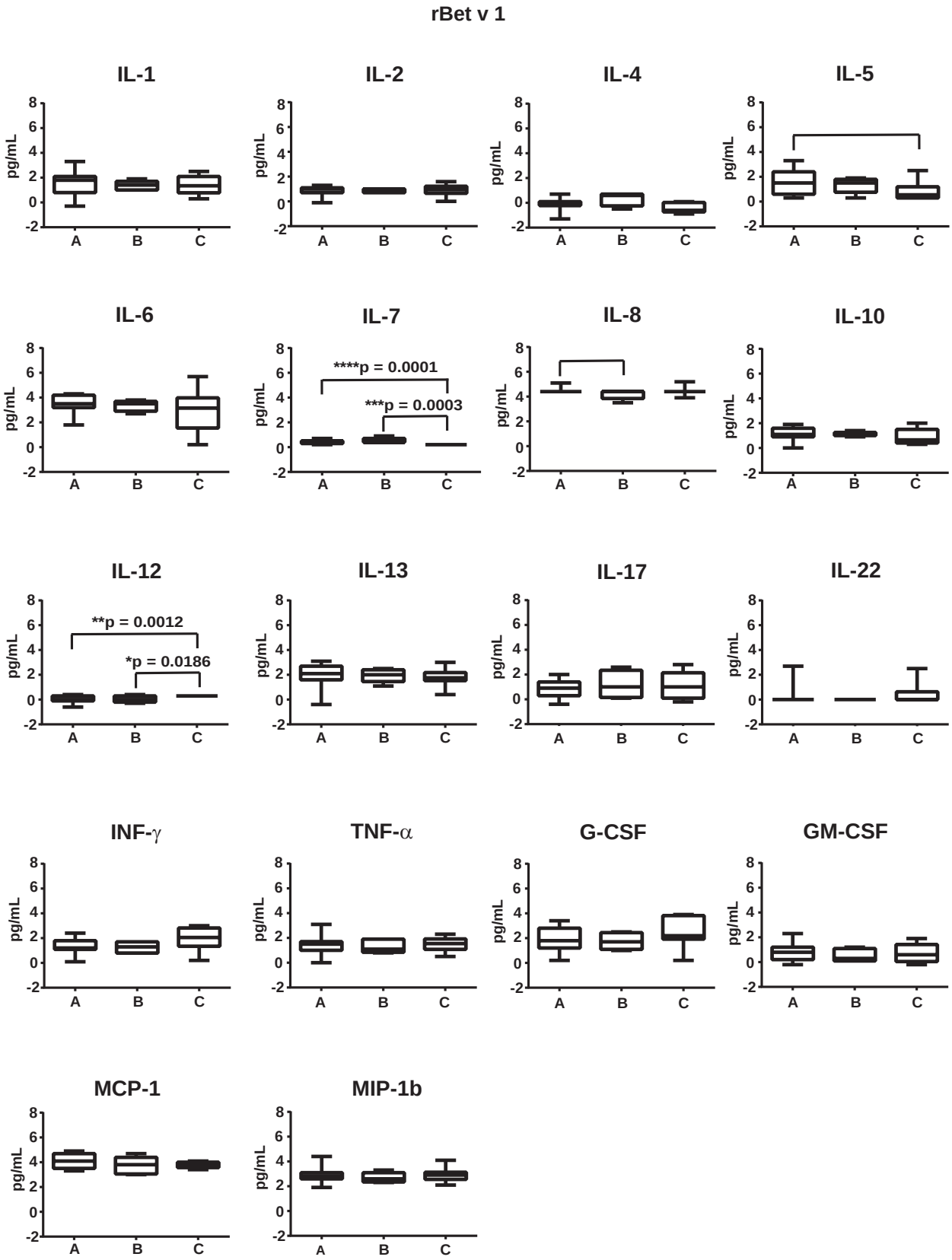

FIG E4. Cytokine levels measured in PBMC cultures on stimulation with rBet v 1 shortly before SPTs and APTs. Shown are cytokine levels (in picograms per milliliter) determined for triplicate cultures as box-and-whisker plots showing minimum, quartiles, median, and maximum values (*y-axes*) for APT-positive patients with birch pollen allergy with positive APT reactions *(A)*, patients with birch pollen allergy with negative APT reactions *(B)*, and subjects without birch pollen allergy with negative APT reactions (*C*; x-axes). Statistically significant differences ($P < .05$) are indicated. *G-CSF*, Granulocyte colony-stimulating factor; *MCP*-1, monocyte chemoattractant protein 1; *MIP-1b*, macrophage inflammatory protein 1.

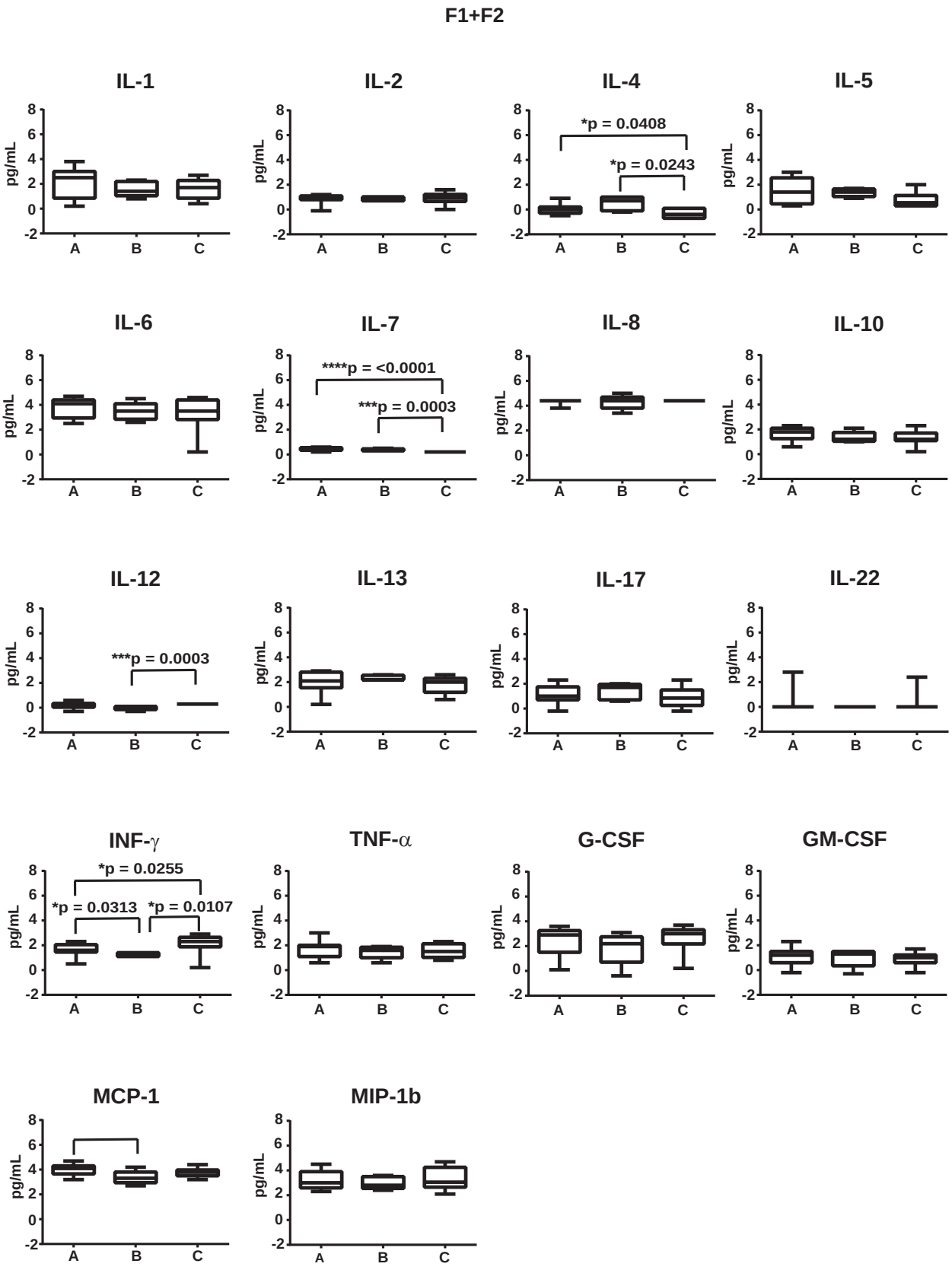

FIG E5. Cytokine levels measured in PBMC cultures on stimulation with the F1+F2 mix shortly before SPTs and APTs. Shown are cytokine levels (in picograms per milliliter) determined for triplicate cultures as box-and-whisker plots showing minimum, quartiles, median, and maximum values (*y-axes*) for APT-positive patients with birch pollen allergy with positive APT reactions *(A)*, patients with birch pollen allergy with negative APT reactions *(B)*, and subjects without birch pollen allergy with negative APT reactions (*C*; x-axes). Statistically significant differences ($P < .05$) are indicated. *G-CSF*, Granulocyte colony-stimulating factor; *MCP*-1, monocyte chemoattractant protein 1; *MIP-1b*, macrophage inflammatory protein 1.

TABLE E1. Clinical and serologic characterization of subjects

						IgE CAP (kU_A/L)		IgE reactivity			
Group	**Subject**	**Sex/age (y)**	**Allergies**	**Symptoms**	**Total IgE (kU/L)**	**Birch**	**rBet v 1**	**rBet v 1**	**F1+F2**	**F1**	**F2**
1	1	F/36	b, a, g, pf, mo, mi	AD, RC, OAS, AS	934	3.52	5.5	+	−	−	−
1	5	F/32	b, a, mi	AD, RC	55.6	0.72	0.76	+	−	−	−
1	7	M/29	b, a, g, pf, mi	AD, RC, OAS	84.2	8	5.76	+	−	−	−
1	8	M/26	b, a, g, mo, mi	AD, RC	590	11.38	12.82	+	−	−	−
1	15	F/26	b, a, g, mo, mi	AD, RC	522	37.4	41.2	+	+/−	−	−
1	18	M/30	b, a, g, pf, npf, mo, mi, w	AD, RC, OAS	4,788	100.2	93.4	+	+	−	−
1	20	M/24	b, a, g, pf, npf, mi, w	AD, RC, OAS	240	31.2	6.62	+	−	−	−
1	21	F/22	b, a, g, pf, mi	AD, RC, OAS, AS	248	7.1	6.78	+	−	−	−
1	22	F/19	b, a, g, pf, npf, mo, mi	AD, RC, OAS	742	46.8	49	+	+	−	−
1	26	M/31	b, a, g, pf, npf, mo, mi, w	AD, RC, OAS	3,198	122.4	125.6	+	+	−	−
1	19	M/22	b, a, g, pf, npf, mo, mi, w	AD, RC, OAS, AS	446	8.26	7	+	−	−	−
1	24	F/44	b, a, g, pf, npf, mi	AD, RC, AS	13,840	52.8	30.2	+	−	−	−
1	25	F/26	b, a	AD, RC, OAS	29	13.24	13.6	+	+/−	−	−
1	27	F/28	b, g, mi	AD, RC	388	36.4	39	+	−	−	−
1	28	M/35	b, a, mo, mi	AD, RC	17.96	0.7	0.7	+	−	−	−
2	9	M/25	b, a, g, pf, mo, mi	RC, OAS	90.8	6.6	6.44	+	+/−	−	−
2	10	M/34	b, a, g, pf, npf, mo, mi, w	RC, OAS	44.6	4.76	3.18	+	+/−	−	+/−
2	13	F/41	b, a, g, pf, mi	RC, OAS	392	47.4	61	+	+	−	−
2	11	M/47	b, a, g	RC, OAS	42	12	13.78	+	−	−	−
2	6	F/25	b, a, g, pf, mi	RC, OAS	412	36	39.4	+	+	−	−
3	2	F/30	g, w	RC	39	<0.35	<0.35	−	−	−	−
3	3	M/33	g, pf, mi, mo	RC	52.6	<0.35	<0.35	−	−	−	−
3	4	M/32	mi	RC	2,552	<0.35	<0.35	−	−	−	−
3	12	M/31	mi	RC	450	<0.35	<0.35	−	−	−	−
3	23	F/28	g	RC	1,708	<0.35	<0.35	−	−	−	−
4	14	F/27	No	−	<2	<0.35	<0.35	−	−	−	−
4	16	F/30	No	−	47.8	<0.35	<0.35	−	−	−	−
4	17	F/33	No	−	<2	<0.35	<0.35	−	−	−	−
4	29	M/23	No	−	21.8	<0.35	<0.35	−	−	−	−
4	30	F/36	No	−	7.1	<0.35	<0.35	−	−	−	−

Allergies: *a*, Animals; *b*, birch; *g*, grass; *mi*, mites; *mo*, molds; *npf*, non–plant-derived food; *pf*, plant food; *w*, weeds.
Symptoms: *AD*, Atopic dermatitis; *AS*, asthma; *OAS*, oral allergy syndrome; *RC*, rhinoconjunctivitis.